PURIFICATION OF ANGIOTENSIN CONVERTING ENZYME INHIBITORY PEPTIDE DERIVED FROM KACANG GOAT MEAT PROTEIN HYDROLYSATE

Jamhari[1], L. M. Yusiati[1], E. Suryanto, M. N. Cahyanto[2], Y. Erwanto[1] and M. Muguruma[3]
[1]Faculty of Animal Science, Gadjah Mada University,
Jl. Fauna No.3, Bulaksumur, Yogyakarta 55281 – Indonesia
[2]Faculty of Agricultural Technology, Gadjah Mada University,
Jl. Flora No.1 Bulaksumur, Yogyakarta 55281 - Indonesia
[3]Faculty of Agriculture, University of Miyazaki,
1-1 Gakuen Kibanadai Nishi, Miyazaki-shi, Miyazaki, 889-2192 - Japan
Corresponding E-mail: jam_hari@ugm.ac.id

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ABSTRAK

Penelitian ini bertujuan untuk mengidentifikasi peptida *Angiotensin Converting Enzyme* (ACE) inhibitor dari hidrolisat protein daging kambing Kacang. Daging kambing Kacang bagian *loin* dihidrolisis dengan enzim *pepsin, trypsin* dan *chymotrypsin.* Hidrolisat protein daging kemudian diuji konsentrasi protein dan aktivitas ACE inhibitor. Peptida ACE inhibitor dipurifikasi melalui serangkaian purifikasi dengan column SEP-PAK Plus C18 Cartridge dan RP-HPLC dengan menggunakan column Cosmosil 5PE-SM, 4,6 x 250 mm. Sekuen asam amino peptida ACE inhibitor kemudian diidentifikasi dengan menggunakan amino acids sequencer. Hasil purifikasi peptida menunjukkan bahwa sekuen asam amino peptida ACE inhibitor dari hidrolisat protein daging kambing Kacang adalah leu-thr-glu-ala-pro-leu-asn-pro-lys-ala-asn-arg-glu-lys. Peptida tersebut mempunyai berat molekul (BM) sebesar 1581 dan berada pada posisi residu ke-20 sampai ke-33 dari protein β-actin daging kambing (*Capra hircus*). Peptida tersebut mempunyai aktivitas ACE inhibitor (IC_{50}) sebesar 190 μg/mL atau 120 μM.

Kata Kunci: Peptida ACE Inhibitor, hidrolisat protein daging, kambing Kacang

ABSTRACT

The objective of this study was to identify the Angiotensin Converting Enzyme (ACE) inhibitory peptide derived from Kacang goat meat protein hydrolysate. Kacang goat meat loin section was hydrolyzed with pepsin, trypsin and chymotrypsin. Protein hydrolysate of Kacang goat meat was then tested the protein concentration and ACE inhibitory activity. ACE inhibitory peptide of the protein hydrolysate was purified through several steps of purification by column SEP-PAK Plus C18 Cartridge and RP-HPLC using a Cosmosil column 5PE-SM, 4.6 x 250 mm. The sequence of amino acid of ACE inhibitory peptide was identified by amino acid sequencer. The results showed that amino acids sequence of ACE inhibitory peptide derived from protein hydrolysate of Kacang goat meat was leu-thr-glu-ala-pro-leu-asn-pro-lys-ala-arg- asn-glu-lys. It had a molecular weight (MW) of 1581 and occurred at the position of 20th to 33rd residues of β-actin of goat meat protein (*Capra hircus*). The ACE inhibitory activity (IC_{50}) of the peptide was 190 μg/mL or 120 μM.

Keywords: ACE Inhibitory peptide, meat protein hydrolysate, Kacang goat

INTRODUCTION

Recent years, peptides from partial enzymatic hydrolysate of food proteins have received greater attention from food scientists than ever before since they posses many benefits. Many biological peptides, with health benefits such as antihypertensive activity, antibacterial activity, mineral-binding activity, enhancement of intestinal activity have been classified and identified from food proteins hydrolysate (Ariyoshi, 1993; Schlimme and Meisel, 1995; Steijins, 1996; Clare and Swaisgood, 2000). These peptides are inactive within the sequence of the parent protein but can be released during enzymatic digestion or food processing (Korhonen *et al*., 1998).

Angiotensin converting enzyme (ACE;

peptidyl dipeptidase, EC 3.4.15.1) plays an important physiological role in blood pressure regulation and in electrolyte and fluid balance (Suetsuna 1998). This enzyme catalyzes conversion of inactive angiotensin I to potent vasoconstrictor angiotensin II by cleaving the dipeptide from the C-terminal of angiotensin I in the rennin angiotensin system, and it inactivates the vasodilator bradykinin in the kallikrein-kinin system. Angiotensin II has several central effects in leading to a further increase in blood pressure. ACE also degrades vasodilative bradykinin in blood vessels and stimulates the release of aldosterone in the adrenal cortex (Cheung *et al*., 1980).

Several inhibitors of ACE have been found to be effective as antihypertensive pharmaceuticals. ACE inhibitory activity in foods has also been studied (Yamamoto, 1997). ACE inhibitory peptides derived from foods, especially milk proteins (casein and whey proteins), have been reported to show antihypertensive effects in spontaneously hypertensive rats (SHR) by oral administration (Maruyama *et al*., 1987; Yamamoto *et al*., 1994; Nakamura *et al*., 1995; Abubakar *et al*., 1998; and Yamamoto *et al*., 1999). The antihypertensive effect of sour milk containing two ACE inhibitory peptides (Val-Pro-Pro and Ile-Pro-Pro) derived from casein was demonstrated in SHR (Nakamura *et al.*, 1995) and in hypertensive patients (Hata *et al*., 1996). Research has also been conducted to characterize the ACE-inhibitory activity derived from other foodstuffs, such as maize (Maruyama *et al*., 1989), fish (Seki *et al*., 1995), fish products (Yokoyama *et al*., 1992; Astawan *et al*., 1995), and eggs (Yoshii *et al*., 1999).

Arihara *et al*. (2001) had successfully identified and isolated peptide inhibitors for ACE from enzymatic hydrolysate of porcine skeletal muscle protein. Two potent ACE inhibitory peptides, mainly found in myosin heavy chain, have been purified from thermolysin digest. Antihypertensive activity of peptides derived from porcine myosin in spontaneously hypertension rat (SHR) was also investigated, and hydrolysate of porcine myosin and peptides showed antihypertensive activity on SHR (Nakashima *et al*., 2002). Purification and characterization of ACE inhibitory peptide derived from porcine troponin C were successfully investigated, and an ACE inhibitory peptide was identified as Arg-Met-Leu-Gly-Gln-Thr-Pro-Thr-Lys (Katayama *et al*., 2003a). The concentration of this nonapeptide necessary to inhibit 50% (IC_{50}) activity of ACE *in vitro* was 34 μM. Peptic hydrolysate of porcine crude myosin also had inhibitory activity with the 50% inhibitory value of 112 μg/mL (Katayama *et al*., 2003b). ACE inhibitory peptide had also derived from porcine skeletal muscle myosin and had antihypertensive activity in SHR (Katayama *et al*., 2007). The peptide was detected as an octapeptide, Val-Lys-Lys-Val-Leu-Gly-Asn-Pro, from 47th to 54th position of myosin light chain. The 50% inhibitory concentration of this peptide was 28.5 μM. However, there has been few studies on ACE inhibitory activity and antihypertensive activity derived from meat proteins, especially from Indonesian local animals. In the present study, the ACE inhibitory peptide derived from Kacang goat meat skeletal muscle proteins had been purified and identified. Such activities and substances could be utilized to produce new healthy meat products, which might open up a new market in the meat industry.

MATERIALS AND METHODS

Samples Collection and Preparation

Materials used in this study were Kacang goat meat loin (*Longissimus dorsi*) obtained from central Java area, pepsin (porcine stomach mucosa), trypsin, chymotrypsin obtained from Wako Pure Chemical Industry Ltd., Japan, ACE from rabbit lung obtained from Sigma Chemical Co. St. Louis, USA), Hippuryl-L-Histidyl-L-Leucine (HHL) free base obtained from Nacalai Tesque, Kyoto, Japan.

Fifty grams of meat with the addition of 100 mL water were blended with a Panasonic food processor for 5 min. The meat extract was then homogenized using Polytron PT-MR2000 for 10 min. The meat extract was incubated into shaking water bath Taitec Personal-11 for 30 minutes at 70^{o}C, and then the extract was cooled with ice.

Hydrolysis protein by pepsin

Meat extract was adjusted into pH 2.0 with 1 M HCl. Pepsin (porcine stomach mucosa) was added into the meat extract at the amount of 0.01 g. After 2 hours incubation at 37^{o}C, the hydrolysate pH was adjusted to 7.0 with 1 M NaOH, and the hydrolysis by pepsin was terminated by heating at 95^{o}C for 10 min, followed by cooling in ice.

Hydrolysis Protein by Trypsin and Chymotrypsin

The trypsin and chymotrypsin were added to the peptic hydrolysate at the amount of 0.01 g each. The peptic hydrolysate was then incubated at 37^{o}C for 2 hours. The hydrolysis by trypsin and chymotrypsin was terminated by heating at 95^{o}C for 10 min, followed by cooling in ice. Protein hydrolysate was then filtered using 1 mL syringe set by a filter (Advantec Dismic-25ES, cellulose acetate 0.45 µM, Toyo Roshi Co., Japan). Filtrate of meat extract was collected for future experiments.

ACE Inhibitory Activity

The assay of ACE inhibitory activity was determined using the method of Cushman and Cheung (1971). ACE, a dipeptidyl carboxypeptidase (E.C.3.4.15.1), extracted from rabbit lung was obtained from Sigma Chemical Co. Hippuryl-L-histidyl-L-leucine (HHL) obtained from Nacalai Tesque Inc., Kyoto, Japan was used as a synthetic substrate. The ACE inhibitory activity was expressed as the 50% inhibitory concentration (IC_{50}) of the hydrolysate in the assay.

Purification of ACE inhibitory peptide

Protein hydrolysate was filtered by SEP-PAK Plus C18 cartridge (Waters Co., Milford, MA, USA), and eluted by 2% acetonitrile (CH_3CN) and 0.1% trifluoroacetic acid (TFA) in water, and 65% CH_3CN and 0.1% TFA in water. The purpose of the step was to remove non protein components in the protein hydrolysate. The filtrate was dried up using freeze dryer, and then diluted with water. Filtrat of protein hydrolysate was then applied into several steps of reverse phase high performance liquid chromatography (RP-HPLC) by using a Cosmosil column 5PE-MS, 4.6 X 250 mm (Nacalai Tesque Co.). First RP-HPLC was eluted by a binary gradient of 0 to 65% CH_3CN and 0.1% TFA in water, with a flow rate of 0.5 mL/min, and wave length of 215 nm. Fractions were collected every 5 min and run for 70 min. Second RP-HPLC was eluted by a binary gradient of 10 to 35% CH_3CN and 0.1% TFA in water, with a flow rate of 0.5 mL/min, and wave length of 215 nm. Fractions were collected every 2.5 min and run for 50 min. Third RP-HPLC was eluted by a binary gradient of 20 to 25% CH_3CN and 0.1% TFA in water, with a flow rate of 0.5 mL/min, and wave length of 215 nm. Fractions were collected every 1 min and run for 30 min. Fourth RP-HPLC was eluted by an isocratic gradient of 15% CH_3CN and 0.1% TFA in water, with a flow rate of 0.5 mL/min, and wave length of 215 nm. Fractions were collected every 1 min and run for 20 min. Fifth RP-HPLC was eluted by the same condition of the fourth RP-HPLC. Fraction showing a single peak was collected and tested on the ACE inhibitory activity.

Amino Acid Sequencing

Fifty µL of fraction showing a single peak was identified the sequence of amino acid by amino acid sequencer in the Laboratory of Shimadzu Technology (Shimadzu Co., Kyoto, Japan).

RESULTS AND DISCUSSIONS

ACE inhibitory activity of protein hydrolysate of Kacang goat meat had been studied and the sequence of ACE inhibitory peptide of protein hydrolysate had been purified and identified. The protein extracts of Kacang goat meat had no ACE inhibitory activity. However, hydrolysis of protein extracts of Kacang goat meat by the pepsin, trypsin and chymotrypsin showed potential ACE inhibitory activity on their hydrolysate with IC_{50} of 316 µg/mL.

The hydrolysate was then applied to SEP-PAK Plus C18 cartridge (Waters Co., Milford, MA, USA) to eliminate the non-protein compounds such as fats and salts. Purified protein was separated using RP-HPLC and eluted with a binary gradient of 0 to 65% CH_3CN . Fraction No.5 of the first RP-HPLC showed the most ACE inhibitory activity with the ACE inhibitory activity of 65.35% (Figure 1). Fraction No.5 was then re-separated with the same column and eluted with a binary gradient of 10 to 35% CH_3CN. Fraction No.5.10 of the second RP-HPLC showed the highest ACE inhibitory activity with the ACE inhibitory activity of 73.49% (Figure 2). Fraction No.5.10 was then re-separated with same column and eluted with a binary gradient of 25 to 30% CH_3CN. Fraction No.5.10.18 of the third RP-HPLC showed the highest ACE inhibitory activity with the ACE inhibitory activity of 81.33% (Figure 3). Fraction No.5.10.18 was then re-separated with same column and eluted with isocratic flow of 15% CH_3CN. Fraction No.5.10.18.9 of the fourth RP-

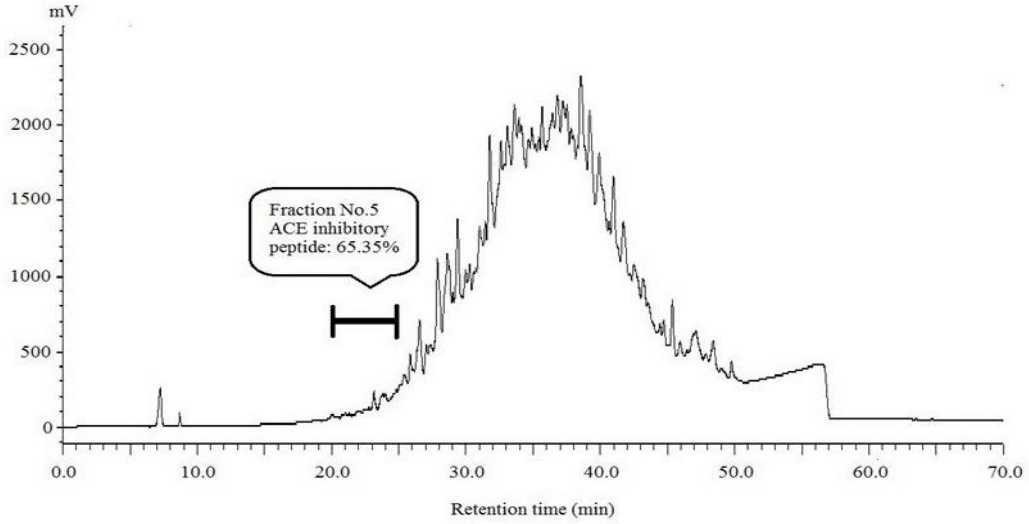

Figure 1. RP-HPLC chromatograms of protein hydrolysates of Kacang Goat meat injected in Cosmosil SPE-MS Column (4.5 x 250 mm) (Nacali Tesque). Protein hydrolysate was eluted with a binary gradient of 0 to 65% CH_3CN and 1% TFA in water, with a flow rate of 0.5 mL/min and wave length of 215 nm. Fraction No.5 showed highest ACE inhibitory activity of 65.35%.

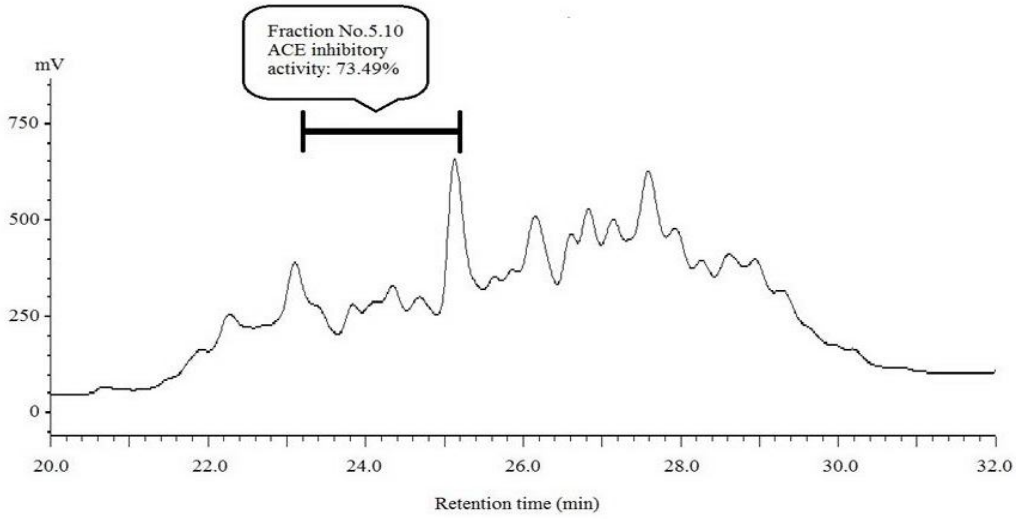

Figure 2. RP-HPLC chromatogram of Fraction No.5 injected in Cosmosil 5PE-MS Column (4.5 x 250 mm) (Nacalai Tesque). Fraction No.5 was eluted with a binary gradient of 10 to 35% CH_3CN and 1% TFA in water, with a flow rate of 0.5 mL/min and wave length of 215 nm. Fraction No.5.10 showed highest ACE inhibitory activity of 73.49%.

HPLC showed the highest ACE inhibitory activity with the ACE inhibitory activity of 88.67% (Figure 4). Fraction No.5.10.18.9 of the fourth RP-HPLC was purified again with the same column and eluted with isocratic flow of 15% CH_3CN. Fraction No.5.10.18.9.9 showing a single peak showed high ACE inhibitory activity with the IC_{50} of 190 µg/mL (Figure 5).

Analysis of amino acid sequence was performed in Shimadzu Technology (Shimadzu Co., Kyoto, Japan). Fraction No.5.10.18.9.9 showing a single peak produced amino acid sequence of LTEAPLNPKANREK (leu-thr-glu-ala-pro-leu-asn-pro-lys-ala-arg-asn-glu-lys) and

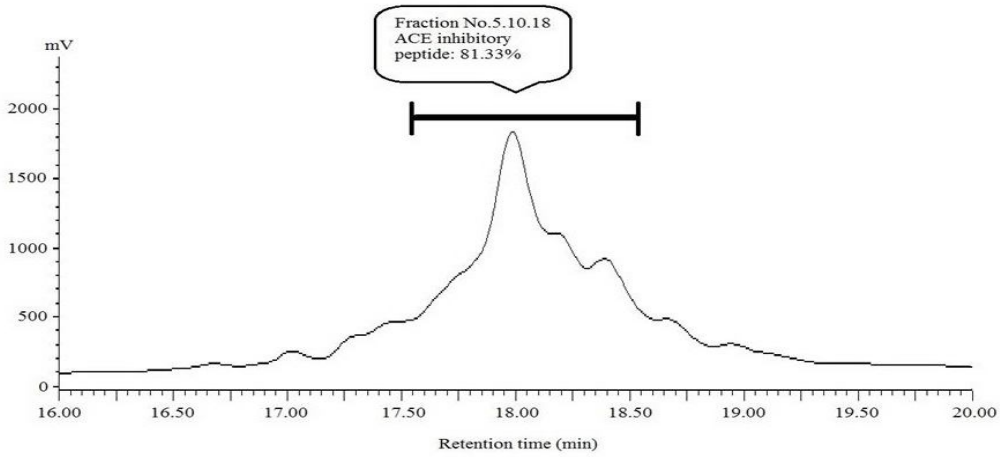

Figure 3. RP-HPLC chromatogram of Fraction No.5.10 injected in Cosmosil 5PE-MS Column (4.5 x 250 mm) (Nacalai Tesque). Fraction No.5.10 was eluted with a binary gradient of 20 to 25% CH_3CN and 1% TFA in water, with a flow rate of 0.5 mL/min and wave length of 215 nm. Fraction No.5.10.18 showed highest ACE inhibitory activity of 81.33%.

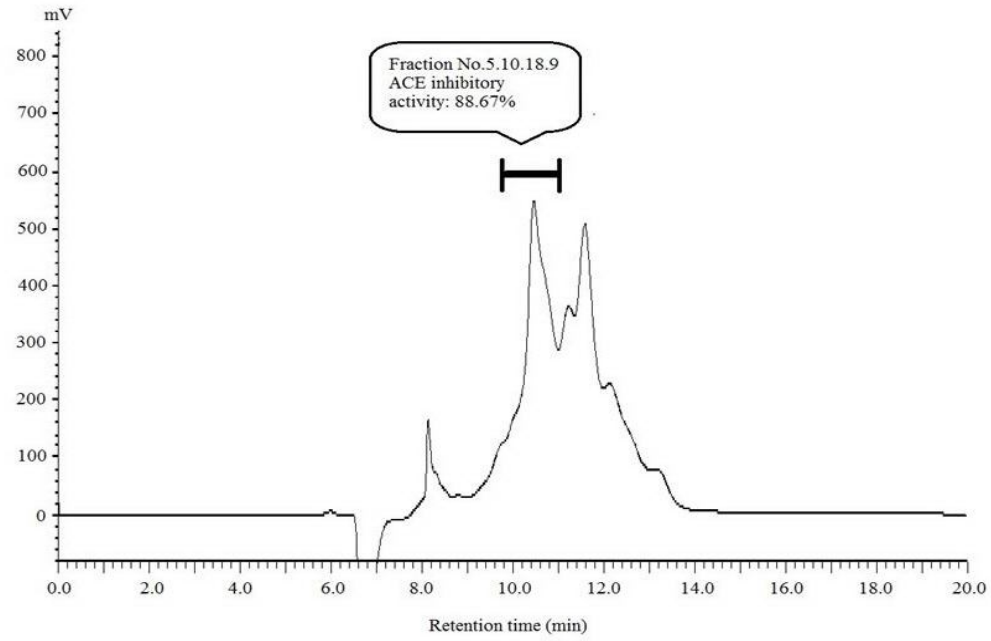

Figure 4. RP-HPLC chromatogram of Fraction No.5.10.18 injected in Cosmosil 5PE-MS Column (4.5 x 250 mm) (Nacalai Tesque). Fraction No.5.10.18 was eluted with isocratic flow of 15% CH_3CN and 1% TFA in water, with a flow rate of 0.5 mL/min and wave length of 215 nm. Fraction No.5.10.18.9 showed highest ACE inhibitory activity of 88.67%.

had a molecular weight or MW of 1581. Based on the molecular weight of this peptide, it can be expressed as IC_{50} of 120 μM. The peptide sequence occurred at the position of 20th to 33rd in the β-actin from goat meat (Figure 6) (NCBI, 2011). β-actin is one of the two non muscle cytoskeletal actins. Actins are highly conserved proteins that are involved in cell motility, structure and integrity (Hanukoglu *et al.*, 1983).

The results showed that the sequence of

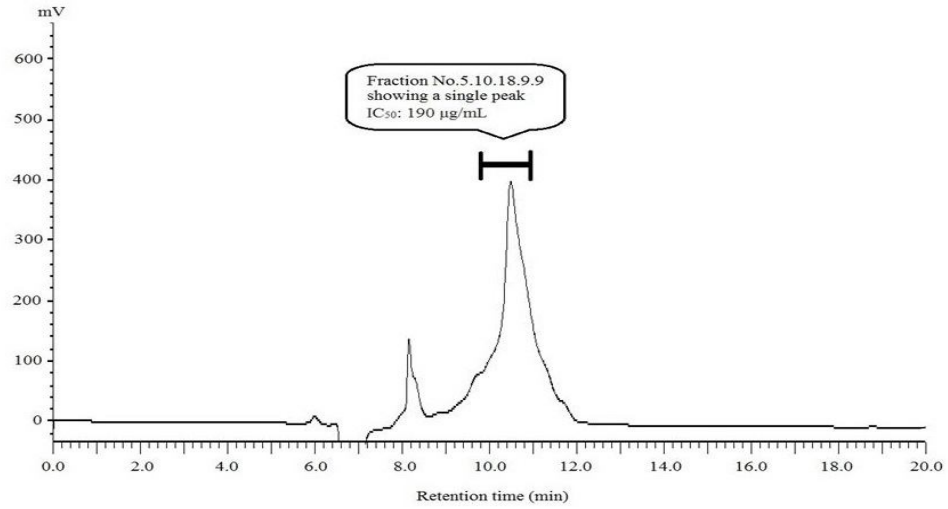

Figure 5. RP-HPLC chromatogram of Fraction No.5.10.18.9 injected in Cosmosil 5PE-MS Column (4.5 x 250 mm) (Nacalai Tesque). Fraction No.5.10.18.9 was eluted with isocratic 15% CH_3CN and 1% TFA in water with a flow rate of 0.5 mL/min and wave length of 215 nm. Fraction No.5.10.18.9.9 showing a single peak showed IC_{50} of 190 µg/mL, was sequenced in Shimadzu Technology (Kyoto, Japan).

1	WHHTFYNELR	VAPEEHPVL**L**	**TEAPLNPKAN**
31	**REK**MTQIMFE	TFNTPAMYVA	IQAVLSLYAS
61	GRTTGIVMDS	GDGVTHTVPI	YEGYALPHAI
91	LRLDLAGRDL	TDYLMKILTE	RGYSFTTTAE
121	REIVRDIKEA		

Figure 6. Amino acid sequences of β-actin of meat goats (*Capra hircus*). ACE inhibitory peptide of LTEAPLNPKANREK had MW of 1581, and occurred at the position of 20th to 33rd residues of β-actin of goat meat protein (*Capra hircus*) (NCBI, 2011)

amino acids of leu-thr-glu-ala-pro-leu-asn-pro-lys-ala-arg-asn-glu-lys can be produced from β-actin of Kacang goat meat protein which was digested by proteases. Chymotrypsin cleaved peptide bound of leu-leu at the position of 19th and 20th residues of goat meat β-actin (Enenkel and Smillie, 1963 *cit.* Beck, 1973), and peptide bound of lys-met at the position of 33rd and 34th residues of goat meat β-actin (Folk and Cole, 1965 *cit.* Beck, 1973). Besides, peptide bound of lys-met at the position of 33rd and 34th residues of goat meat β-actin was also cleaved by trypsin (Green and Naurat, 1954 *cit.* Beck, 1973). Thus, the ACE inhibitory peptides can also be produced from processed meat products. Digestive enzymes such as pepsin, trypsin, and chymotrypsin in the digestive tract can result in ACE inhibitory peptides of muscle protein (Arihara *et al.*, 2001). Therefore, ACE inhibitory peptides can be produced in the digestive tract from meat consumed. Bioactive components including ACE inhibitory peptides had potential health benefits, and they can be introduced into meat products, thus the value of meat products can be improved. The use of ACE inhibitory peptides from meat protein can produce healthier meat products.

CONCLUSION

Antihypertensive peptide has been isolated from Kacang goat meat protein hydrolysate. The antihypertensive peptide sequence was leu-thr-glu-ala-pro-leu-asn-pro-lys-ala-arg-asn-glu-lys, had a molecular weight (MW) of 1581, and occurred at the position of 20th to 33rd residues of β-actin of goat meat protein (*Capra hircus*). The ACE

inhibitory activity (IC_{50}) of the peptide was 190 μg/mL or 120 μM.

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